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Distribution and Prevalence *Potato virus Y* Isolates Obtained from Potatoes Grown in the Iran by RT-PCR

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Abstract

To survey the distribution and prevalence of *potato virus Y* (PVY) strains in north western Iran, 381 symptomatic infected samples of the main potatoes grown in the said region were collected from the fields between (Hall et al., 1998) 2007 and 2008. The collected samples were first tested for PVY infection via a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) technique. Of the total number of collected samples, 79 (20.73%) tested positive for PVY infection. The highest level of PVY infection was observed in the district of Gilak-Abad in Sarab County, whereas the lowest infection was observed in the district of Oughan in the suburbs of Sarab city. The RT-PCR detection of PVY strains by using specific primers resulted in the amplification of DNA fragments specific to the PVY strains NTN, C, O, and N at 725, 1553, 352, and 616 bp, respectively. The highest strain diversity of PVY was detected in the district of Shirehjin in Sarab city and the lowest in the district of Ghaleh Jugh in Bostan-Abad city. Both single and multiple infection types of the PVY stains were observed in the region. Of the 79 PVY-infected samples, 77.21% were infected with strain O, 62.02% with strain C, 39.24% with strain N, and 8.86% with strain NTN. The highest level of multiple infections was observed in the combinations of the strains C+O (27.84%) and the triple strains O+N+C (15.18%). This paper is the first to report the detection of the PVY strain NTN in Iran.

Keywords: RT-PCR, Molecular diagnosis, Potato, ELISA, *Potato Virus Y* (PVY)

1. Introduction

Potato virus Y (PVY, Potyviridae: Potyvirus), one of the most common and destructive viruses found in potatoes (Solanum tuberosum L.), immensely impacts the potato industry because it causes tuber necrosis, which leads to(Sigvald, 1992); (Hall et al., 1998). The viral genome includes a single-stranded ss plus, an RNA molecule about 10 kb in length, a VPg protein covalently attached to its 5´ end, and a poly-A tail at its 3 end. The viral RNA encodes a single large polypeptide, which is cleaved by three virus-encoded proteases into nine products (Dougherty and Carrington, 1988). Different potato PVY isolates from the outcrop can be categorized into four groups based on virulence and host response: PVYO, PVYN, PVYNTN, and PVYNW O-Strain (Ordinary) (Glais et al., 2005). The last group, also termed as PVYNW or PVYN:O, is the common group that can be detected by O- but not by N-specific monoclonal antibodies (MAB). PVYN or the venial necrosis group induces PVYNW in North America and PVYNTN (Chrzanowska, 1991, Glais et al., 2005); (Nie et al., 2004, Nie and Singh, 2003). PVYO is also known as the common group, PVYN the tobacco venial necrosis group, PVYC the stipple streak group, and PVYNTN the tuber necrosis strain group. PVYNTN induces the potato tuber necrotic ring spot disease. Other groups have also been identified, including PVYZ and a new group that induces venial necrosis in tobacco and possesses the coat protein gene typical of O-strains (Solomon-Blackburn and Barker, 2001); (Blanco-Urgoiti et al., 1998); (Jones and Orr, 1994). This latter group can be detected by O- but not by N-specific monoclonal antibodies. This variant, also known as PVYN:O, is termed as PVYNW in North America (Nie et al., 2004). Some workers (Glais et al., 2005) consider PVYNW and PVYNTN as sub-strains of PVYN. The symptoms induced by the various strain groups in potatoes depend on the particular isolate and host. Typically, the foliage symptoms of PVYO, PVYC, PVYN, PVYNW, and PVYNTN include mild to severe leaf

mosaic, rugosity, crinkling, severe systemic necrosis, dropping of leaves (leaf drop streak) and dwarfing, mild leaf symptoms (which may not often be obvious in infected plants), and severe leaf symptoms. Different structures of PVY variants have been defined by (Lorenzen et al., 2007). For the epidemiological investigation of PVY, in-the-frame and in-the-field evolution reliable test systems, like serological tests and enzyme-linked immunosorbent assays (ELISA), are necessary. Although ELISA has been adopted for routine testing, it is not sufficiently sensitive. Thus, the search for more sensitive techniques remains a challenge. In this respect, RT-PCR tests that combine cDNA synthesis and PCR amplification have been proposed for the detection of potato viruses like PVYO and PVYNTN. In this test, crude sap from the leaves are used directly in the RT-PCR reaction. In our study, ELISA was used as the basic method because it is simple, sensitive, and generally adequate for the large-scale screening of infected plants. The results of ELISA were combined with those from dot-blot ELISA. Lastly, the PVY strains were distinguished via PCR. The aim of the tests was to correlate the appearance of the symptoms with the prevalence of PVYNTN, PVYN, PVYO, and PVYC in the potato fields of northwestern Iran.

2. Methodology

2.1 Virus source and plant material

A total of 381 potato leaf samples were collected from selected fields in northwestern Iran from 2007 to 2008. The samples were collected mainly by the authors. The leaf samples were submitted to our laboratory. All original samples were initially tested via serological and RT-PCR assays and then stored in the freezer at a temperature of -80 °C. Of the 381 original samples, 79 were further selected based on serological properties, molecular analysis, and representativeness of the geographic region for inoculation into *N. tabacum cv. Samsun*. They were subsequently characterized via serology, RT-PCR, and bioassays.

2.2 Virus isolates

The virus isolates were provided by Dr. Reza Porrahim of the Iranian Plant Protection Biodiversity and Genetic Resource Complex. The isolates were maintained in *N. tabacum cv. Samsun*. The leaves of the infected plants were harvested two weeks after inoculation and were tested via ELISA for the occurrence of other potato viruses by using cultured polyclonal antibodies against PVA, PVX, and PVS. In addition, RT-PCR was also performed for the PVY strain.

2.3 Serological testing—ELISA

DAS-ELISA was conducted following the methods of Clark and Adams (Clark et al., 1976). For the present study, commercial polyclonal antibodies against PVY and polystyrene micro-titre plates were used. The plant sap was diluted to 3 mL in phosphate-buffer saline with 2% PVP to test the inhibition of the antigen-antibody reaction. All samples were tested in triplicate by using pairs of wells containing 200 mL of the diluted extracts. The uninfected potatoes were also included in ELISA as negative control samples. The absorbance at 405 nm was measured for 5, 2, and 4 h after incubation with the substrate by using an ELISA reader. The data were processed using SPSS.

2.3 Partial purification of PVY particles

Three samples of PVY isolates were selected from the fields of every province covered in the study. They were mechanically inoculated into *tobacco cv. Samsun* and *Chenopodium amaranticolor* to determine their different strains. All plants were grown in a greenhouse at 18 °C to 22 °C with 12 h of light. Triplicates of every plant at the three-leaf stage were lightly sprinkled with carborundum powder prior to mechanical inoculation with PVY potato leaf extracts, which were ground with a mortar and pestle in an extraction buffer at a 1:10 ratio. About 7 to 10 days post inoculation, foliar symptoms were recorded daily for the next 15 to 20 days. The development of their symptoms was compared with positive and negative control plants. The differential plant used for the detection of every strain of PVY showed different symptoms, which were identified via RT-PCR for the corroboration of every sample.

2.4 Total RNA extraction

RNA was extracted from potato leaves via a slightly modified version of the method of (Mekuria et al., 2003). The tissues of the potato leaves (200 mg to 300 mg) were frozen in liquid nitrogen and ground to a fine powder to produce a leaf homogenate. About 3 mL of the extraction buffer (EDTA 0.01M, Lich 0.1 M, Tris-HCl 0, 1 M, SDS 1%, LiCl 4M, PVP 5%, [w/v]) and sodium metabisulfite 2% (w/v) were added and mixed. About 700 µL of the sample was decanted into a 1.5 mL microfuge tube. About 800 µL of phenol-chloroform-isoamyl alcohol (1:24:25) was added and mixed. The tube was incubated in ice for 15 min before centrifugation for 15 min at 14000 rpm and 4 ºC. About 400 µL of the supernatant was transferred to a fresh microfuge tube and an equal volume of 4 M LiCl was added. All samples were kept at -20 ºC for 4 h following centrifugation for 15 min at 14000 rpm and 4 ºC. The supernatant was extruded. About 400 µL of distillated water, 800 µL of cold ethanol, and 40 µL of sodium acetate 3 M were added into every tube, all of which were centrifuged again for 15 min at 14000 rpm and 4 ºC. The supernatant was discarded again. For last time, the microfuge tube was washed with 100 µL of cold ethanol (70%) and centrifuged for 5 min at 4000 rpm and 4 ºC. The supernatant was excreted and the tube was air-dried. Finally, the tube was re-suspended in 30 µL of RNase-free water and stored at -20 ºC.The RNA concentration was calculated by measuring its absorbance at 260 nm.

2.5 RT-PCR amplification

About 0.1 µL to 5 µL of the total concentrated RNA extract and 0.2 µL of a random hexamer were diluted with RNase-free water until they reached a concentration of 11 µL to reduce nonspecific amplification. The extract was incubated at 70 °C for 15 min and chilled in ice for 3 min. About 4 µL of the reaction buffer (5X), 2 µL of each dNTP mix (10 mM), 0.5 µL of ribonuclease inhibitor, and 12.5 µL of distillate H_2O were added to the denatured RNA extract until the mixture reached a volume of 25 µL. The mixture was incubated for 5 min at 25 ºC. A total of 200 units of reverse transcriptase was added following incubation for 10 min at 25 ºC and 60 min at 42 ºC. The RT reaction was terminated at 70 ºC for 10 min. The resulting cDNA was stored at -20 °C by using previously reported primer sequences and reaction conditions.

To distinguish the PVYO, PVYC, PVYN, and PVYNTN isolates, PCR was performed using 2 µL of the cDNA mixture at a final volume of 25 µL containing 2.5 µL of 10× PCR buffer. About 1.7, 2 µL of $MgCl_2$ for PVYO, PVYN and PVYO, PVYNTN were set up respectively. In addition, 1 µL of dNTP (4 Mm), 1 µL of the reverse primer (10 µM) and 0.5 unit of *Taq* DNA polymerase. PCR was conducted in a thermocycler machine supplied by Eppendorff. The annealing temperatures used for the PVYN, PYVO, PVYC, and PVYNTN isolates were 56 ºC, 58 ºC, 54 ºC, and 60 ºC, respectively, in the first cycle. A temperature of 94 ºC (1 min) was used in the next 35 cycles; 54 ºC (30 s) in the succeeding 35 cycles; and 72 ºC (90 s, 10 min) for the last 35 cycles and one final cycle. The PCR products were fractioned and assessed on a 1.5% (w/v) TAE agarose gel at 110 V for 25 min. They were kept in ethidium bromide (0.5 µg/mL) for 20 min and photographed with UV illumination with imaging system.

2.6 Primer design

The sequences of the primers for PVYN, PVYO, and PVYC were provided by Dr. Farshad Rakhshandehroo from Islamic Azad University, Science and Research Branch Faculty of Agriculture and Natural Resources, Tehran, Iran. The primers included the reverse primer 5´^{1780}CCGAATGGGACAAGAAAACTTG^{1822} 3´, which is located in the coat protein gene. The forward primer 3´ ^{2505}GCAGTTTTAGCGCTGACTC ^{2524}5´ gene position was named PVYN and had a product length of 725 bp. The reverse primer 5´ ^{1005}AATTGTACGATGCACGTTCTAGA^{1028} 3´ and the forward primer 3´ ^{2558}GGCTCATCTAACAGCAACTGTC^{2580} 5´ were named PVYO and had a product length of 1553 bp. The reverse primer 5´ ^{460}CAGCCATCTGAAAGTAGTGC ^{480}3´ and the forward primer 3´ ^{125}TTGAAAACCGTCTTAGTTAGTT^{147} 5´ gene position were named PVYC and had a length of 335 bp. The reverse and forward primers for PVYNTN were 5´ TGATGAARTTGAGTGCGATAC 3´ and 3´ CATCGCGCAGTTACGAAC 5´, respectively. Their gene positions were 8535–8556 and 9151–9169, respectively, because of their length, which is 616 bp. PVYNTN was designed based on the conserved sequence of the PVY Genbank databases (Table 1).

Table 1. Primer and probe sequences and optimized concentration used in RT-PCR reaction for catalase different strains of PVY (Potato Virus Y)

Name	Sequence	Name of target	Product length
YN3-2505 (b)	GCAGTTTTAGCGCTGCTGACTC	PVY^{N}	725 (bp)
YN5-1780 (b)	CCGAATGGGACAAGAAAACTTG	PVY^{N}	
YO3-2558 (c)	GGCTCATCTAACAGCAACTGTC	PVY^{O}	1553(bp)
YO5-1005 (c)	AATTGTACGATGCACGTTCTAGA	PVY^{O}	
YC3-460 (h)	CAGCCATCTGAAAGTAGTGC	PVY^{C}	335 (bp)
YC5-125 (h)	TTGAAAACCGTCTTAGTTAGTT	PVY^{C}	
YNTN-8535	TGATGAARTTGAGTGCGATAC	PVY^{NTN}	616 (bp)
YNTN-9151	CATCGCGCAGATTACGAAC	PVY^{NTN}	

F=Forward
R=Reverse

2.7 Tobacco and potato bioassay

A total of 27 PVY field isolates were selected based on their serological analysis with agent geographical location. They were mechanically inoculated into *N. tobacco cv. Samsun* and *Chenopodium amaranticolor* to determine their pathotype. All plants were grown in a greenhouse at 15 °C to 18 °C with 12 h of light. The tobacco plants were in their three-leaf stage and were lightly sprinkled with carborundum powder prior to mechanical inoculation with PVY potato leaf extracts, which were ground with a mortar and pestle in an extraction buffer (0.01 M sodium phosphate, pH 7.5, containing 0.4% sodium sulfite) at a 1:10 (wt/vol) ratio. About 7 to 10 days post inoculation; the foliar symptoms manifested and were recorded daily for the next 15 to 20 days. The development of the symptoms of the uninoculated control plants and that of the symptoms of the plants inoculated with the control isolates PVYO, PVYN, and PVYC were compared. However, the PVYNTN was identified only through a comparison of its clear symptom with the original symptom and through molecular assessment (McDonald and Singh, 1993, Lorenzen et al., 2006).The plants were monitored for the foliar symptom development characteristic of PVY (mosaic, venial necrosis, spot necrosis, and leaf necrosis). All tobacco and *Chenopodium amaranticolor* plants that were mechanically inoculated were tested approximately 3 to 4 weeks after inoculation via RT-PCR to confirm their infection status.

RESULTS

Serological identification of viral isolates

Of the 381 isolates tested by ELISA, a total of 79 samples were found infected with PVY. In this assessment, each area (Bostan Abad, Shirehjin, Gilak Abad, Karjan, Oghan, Razeligh, Khalil Abad, Taran, and Bahreman) showed different distributions of PVY infection (Table 2). Gilak Abad showed the highest percentage (27%) of infection. Shirehjin, Razeligh, Bhreman, Khalil Abad, Bostan Abad, and Oghan showed 22%, 22%, 22%, 19%, 18%, and 16%, respectively. Taran did not show any infection.

Table 2. Single, Double and Triple Strain infection in different Geographical Place (Bostan abad, Shirehjin, Gilak Abad, Karjan, Oghan, Razeligh, Khalil Abad, Taran and Bahreman) in Iran.

Strains	Type of infection		Single Strain infection				Double Strain infection				Triple Strain infection		
Geographical place	Total samples of each era	Number of samples infected with PVY	O	C	N	O	O+N	O+C	N+NTN	O+N	O+C+NTN	O+N+NTN	C+O+N
Bostan abad	9	48	1	1	-	1	+	++++	-	+	+	+	-
Shirehjin	20	88	2	2	1	2	+++++	+++++	+	+++++	++	-	++
Gilak Abad	10	37	2	1	-	2	+	++++	+	+	-	-	+
Karjan	13	60	3	3	4	3	-	++	-	-	-	-	+
Oghan	8	49	2	1	-	2	-	+++	-	-	-	-	++
Razeligh	9	40	1	1	-	1	++	++	-	++	-	-	+++
Khalil Abad	6	31	1	-	-	1	-	+	-	-	++	-	+
Taran	-	10	-	-	-	-	-	-	-	-	-	-	-
Bahreman	4	18	1	-	-	1	-	+	-	-	-	-	++
Total	79	381	13	9	5	13	9+	22+	2++	9+	5+	1	12
Percentage of each infection	20.73		16.45	11.39	6.32	16.45	11.39	27.84	2.53	11.39	6.32	1.26	15.18

Detection of PVY strains

The PVY assay was performed using well-characterized isolates from each of the four strains. The results showed that the assay performed as expected, and the primers for each strain amplified the corresponding isolates (Fig 1,2,3,4). Competitive bands were present where expected (e.g., isolates of N, NTNT, C, and O with each of the primers), the efficient amplification of which ensured robust strain discrimination. The assay gave equivalence results when the isolates were tested using the immunocaptured leaves of *N. tobacco cv. Samsun* and *Chenopodium amaranticolor* that were used for the biological assessment. All of samples collected were isolated based on the field of origin.

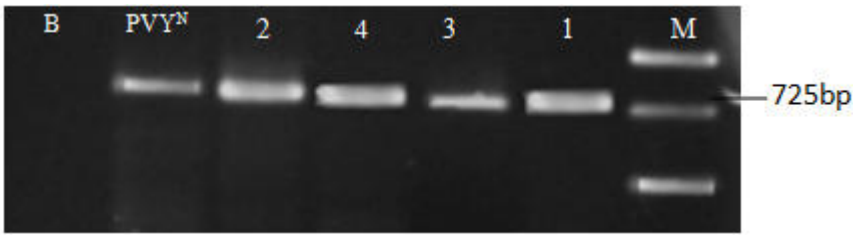

Figure 1. PCR detection of PVY^{N} Specific 725bp from 1:Bostan abad, 2:Sarab, 3:Tabriz, 4:Ardabil, B-Negative control, M-Marker and Positive control.

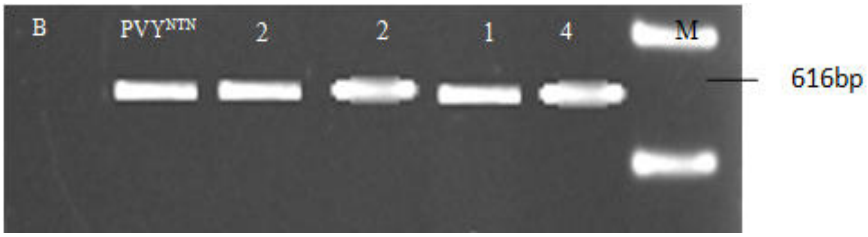

Figure 2. PCR detection of PVY^{NTN} Specific 616bp from 1:Bostan abad, 2:Sarab, 4:Ardabil B-Negative control, M-Marker and Positive control.

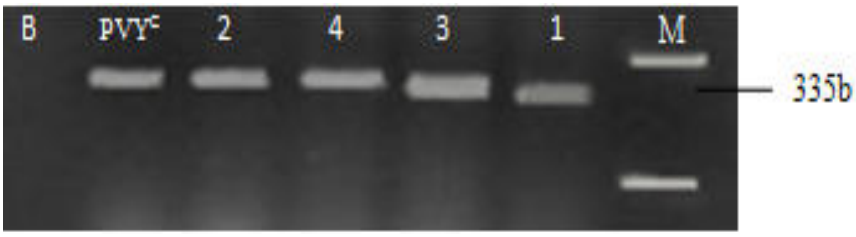

Figure 3. PCR detection of PVY^{O} Specific 335bp from 1:Bostan abad, 2:Sarab, 3:Tabriz, 4:Ardabil, B-Negative control, M-Marker and Positive control.

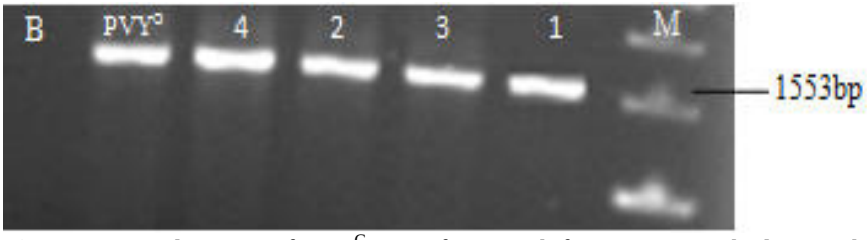

Figure 4. PCR detection of PVY^{C} Specific 1553pb from 1:Bostan abad, 2:Sarab, 3:Tabriz, 4:Ardabil, B-Negative control, M-Marker and Positive control.

Geographic origins of strains

Of the 79 PVY strains obtained from different areas in Iran, most of the strains belonged to the various PVYO serotypes. About 77.21% of the infected samples came from Shirehjin, which showed the highest infection for this strain. By contrast, Taran showed no infection for this strain. The next more common strain belonged to the PVYC serotypes. Again, most of the infection, 62.02% of the infected samples, came from Shirehjin. By contrast, Khalilabad showed the lowest infection for PVYC. Taran did not show any putrefaction and was the place of origin of only 39.24% and 8.86% of the infections related to PVYN and PVYNTN, respectively. Based on this study's observations, the samples from Shirehjin had all strains related to PVNTN, the first reported strain of this kind in Iran, and characterized as at least local to Shirehjin. By contrast, all the samples from Karjan, Oghan, Razeligh, Taran, and Bahreman tested negative for the PVYNTN strain (Table 3). Based on the results, the samples exhibited a mixed infection for all strains. More specifically, the samples from different geographical areas showed single, double, and triple infection. In the samples with single infection, PVYO showed the largest distribution. In the samples with double infection, PVYO:C showed the largest distribution at 27.84%, whereas PVYN:NTN showed the smallest distribution at 2.53%. PVYC:O:N indicated the highest putrefaction at 15.18%. In the samples with triple infection, PVYO:N:NTN showed the least distribution (Table 3).

Table 3. Geographic sources and original hosts of potato virus Y and percentage of single, multiple and triple infections of different strains of potato virus Y in Iran.

Origen area	Number of sample	Number of sample with PVY* infect	Percentage of samples infect with PVY*	Number of sample with PVY^{O} infect	Number of sample with PVY^{C} infect	Number of sample with PVY^{N} infect	Number of sample with PVY^{NTN} infect
Bostan Abad	48	9	18.75	8	6	2	2
Shirehjin	88	20	22.72	16	11	9	3
Gilak Abad	37	10	27.02	8	6	3	1
Karjan	60	13	21.66	6	6	5	-
Oghan	49	8	16.32	7	6	2	-
Razeligh	40	9	22.5	7	7	5	-
Khalil Abad	31	6	19.35	6	3	4	1
Taran	10	-	-	-	-	-	-
Bahreman	18	4	22.22	3	4	1	-
Total	381	79	170.54	61	49	31	7
Percentage		20.73	18.94	77.21	62.02	39.24	8.86

PVY* = Potato Virus

DISCUSSION

PVY is a diverse virus species. The separation and classification of PVY isolates into pathotypes or strains have proven to be challenging. Biological properties, serological reactivity with ELISA, and molecular measurement via RTPCR can be used to differentiate the virus strains into four groups: PVYC, PVYO, PVYN, and PVYNTN. The first three strains are more widespread. The last strain is rarer. Its report in this paper is the first in Iran. Nevertheless, the distinction between the strains is contractual. This study attempted to define the all strains via biological identification by using indicator plants in the greenhouse and the specific conditions of the manifested symptoms. Molecular technology was also employed for a more accurate biological identification and determination of the distribution of PVY strains. Our efforts were focused on determining the pathogenic nature of all the PVY strains. The first aim of this study was to characterize and differentiate PVY isolates obtained from various potato-producing regions in Iran. The second aim of this study was to determine the distinctions among the different strains of PVY. At present, the results of our study clearly yielded limited information on the PVYNTN and PVYN strains. More data related to PVYC and PVYO were obtained. This study also showed the present state of two or three infections of every strain (PVYO:C, PVYN:NTN, PVYC:O:N, and PVYO:N:NTN) from different potato-producing regions.

Based on the previous findings of Mialec et al. (2001), PVYN has also been identified in the United States. Specifically, the distribution of this stain has been reported in California and Florida. PVYN was also isolated from tobacco in Canada, Prince Edward Island, New Brunswick, Nova Scotia, Quebec, and Ontario. However, at around the same time when these findings were published, PVYN was not found in western Canada (Milavec and Chapuis, 2004, Moravec et al., 2003)

According to Hinrichs *et al*., 1998 (Hinrichs et al., 1998), the geographical distribution of PVYN is more limited than that of PVYO. The distribution of PVYC is more restricted than that of either of PVYN and PVYO. The characterization of the members of PVYC group is unclear, although many cultivars are hypersensitive to strains of PVYC (Singh et al., 2003).

In North American literature, PVYN and PVYO are sometimes designated as recombinant strains (PVYN:O) or recombinant NTN strains. The structure of the North American N and NTN variants differ from that of the European recombinant variants. Given the wide variation in the climatic conditions of potato-producing regions, the variation of PVY strains is not surprising. Furthermore, the different cultivars of potato, each with different levels of resistance and susceptibility, are important issues related to infection and distribution. The host selection or non-selection of PVY, such as tomato, tobacco, and other plants, also have an important effect on the long-term movement of the different strains of PVY and on the rearrangement of new strains. Although

certified seeds have much less virus than table stocks (Singh et al., 2003), seed potatoes still represent a potentially important source of new viral strains.

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